

# Modulation of Elastase Binding to Elastin by Human Alveolar Macrophage-derived Lipids

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Human neutrophil elastase (HNE), an enzyme secreted by activated neutrophils, can bind to and degrade extracellular matrix including human lung elastin. This protease is believed to play an important role in several destructive processes including pulmonary emphysema. In this study, we hypothesized that an alveolar macrophage (AM) product or products may interact with neutrophil elastase (NE) and modulate its binding to elastin. Elastase binding to elastase was evaluated by a modified elastase functional assay using a synthetic substrate. Supernatants from cultured AM inhibited elastase binding to elastin at a dose-dependent manner without inhibiting functional elastase activity. The AM products had a heterogeneous molecular weight ranging from 440,000 to 54,000. The activity was heat-stable, but was lost after ultracentrifugation. After lipid fractionation, neither the aqueous nor the lipid fractions contained activity, suggesting that the factor may be a lipid complex. Culture supernatants from smokers' AM released significantly higher amounts of the factor than nonsmokers. In addition, high-molecular-weight elastase was present in bronchoalveolar lavage fluid (BALF) obtained from patients with pneumonia. Most of the *in vivo* high-molecular-weight elastase was lost after lipid extraction. In conclusion, macrophages release a factor or factors, probably lipid, which can interact with NE and inhibit its binding to human lung elastin without inhibiting elastase activity. This macrophage-derived factor may play a role in protecting the lung from NE by partitioning elastase into the airspace and thus protecting the interstitial connective tissue matrix from elastase degradation. Fujita J, Sköld CM, Daughton DM, Ertl RF, Takahara J, Rennard SI. Modulation of elastase binding to elastin by human alveolar macrophage-derived lipids.

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Neutrophil elastase (NE), the major protease released by neutrophils, can bind to and degrade extracellular matrix in the lung, including lung elastin (1, 2). In emphysematous lung, extracellular elastase has been described in association with elastic fibers (3). Because the binding of elastase to elastin is very tight, it is likely that the interstitial elastic fiber acts as an affinity matrix and concentrates the enzyme within the alveolar interstitium (3). However, in the airways of patients with acute pneumonia, where free elastase activity is readily detected, destruction of interstitium is usually minor, suggesting that mechanisms exist to protect the lung from proteolytic attack.

The alveolar macrophage (AM) is the major mononuclear phagocyte present in the lower respiratory tract. It is believed

to play an important role in directing and modulating inflammatory processes (4). Emphysema is a disease associated with smoking and because smokers have an increased number of AM in their bronchoalveolar lavage fluid (BALF) (5, 6), we have hypothesized that AM products could interact with other inflammatory mediators. Specifically, we asked whether products from macrophages could modify the binding of elastase to elastin. To test this hypothesis, elastase binding to elastin was quantitatively evaluated using a modified elastase functional assay. Our results suggest that macrophages are capable of releasing a factor, likely a lipid, which can bind elastase and prevent the binding of this protease to elastin.

## METHODS

### Study Populations

Seven healthy nonsmokers (5 male, 2 female, age  $27.6 \pm 1.1$ ; mean  $\pm$  SE), and 13 healthy smokers (8 male, 5 female, age  $35.6 \pm 2.3$ ) were studied. The smokers had  $39.3 \pm 4.0$  pack-years of smoking history. The study subjects had no history or physical findings suggesting lung disease, and they all had normal chest roentgenograms and normal lung function tests. Bronchoscopy and bronchoalveolar lavage (BAL) were also performed for clinical reasons on patients with pneumonia. All subjects were studied under University of Nebraska Institutional Review Board approved guidelines after informed consent had been obtained.

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### BAL and Macrophage Culture

BAL was performed with an Olympus fiberoptic bronchoscope as previously described (7). Briefly, after local anesthesia, the bronchoscope was gently wedged in a subsegmental bronchus. Lavage was performed by infusing 20-ml aliquots of sterile saline into each of three lobes and immediately aspirated. The final  $4 \times 20$ -ml aliquots, which represent predominantly alveolar material (8), were pooled and used in the present experiments. After centrifugation, the BALF was stored at  $-80^\circ\text{C}$  until used. BAL cells were washed four times with Hanks' balanced salt solution, and resuspended in RPMI-1640 medium at a concentration of  $10^6$  cells/ml. Cells were plated at  $1 \times 10^6$  cells/ml in RPMI-1640 without serum and incubated in 5%  $\text{CO}_2$  at  $37^\circ\text{C}$  in 6-well tissue culture plates (Falcon; Beckton Dickinson Labware, Lincoln Park, NJ). The supernatant medium was aspirated after 24 h culture and frozen at  $-80^\circ\text{C}$  until use. Cell differential count on a Diff-Quik stained cytocentrifuge preparation revealed that  $> 95\%$  of the cells had morphological features of AM.

### Elastin Binding Assay

To quantify elastase binding to elastin, a modified elastase functional assay was used (9). Microtiter plates were coated with  $4 \mu\text{g/ml}$  of human lung elastin (Elastin Product Company, Pacific, MO; lot 86709). In parallel experiments, wells were also coated with type IV collagen. The coated plates were washed three times with 20 mM phosphate-buffered saline (PBS), pH 7.4, with 0.025% Tween 20 (PBS-Tween). A volume of 100  $\mu\text{l}$  of sample was incubated for 1 h at room temperature with 1  $\mu\text{g/ml}$  of human neutrophil elastase (HNE) (Elastin Product Company; lot 86091) dissolved in PBS-Tween. After incubation, these solutions were transferred to the elastin-coated plates and incubated for 30 min at  $+4^\circ\text{C}$ . After washing three times with PBS-Tween, elastase substrate solution methoxysuccinyl-L-alanyl-L-alanyl-L-prolyl-L-valine *p*-nitroanilide (Sigma Chemical Company, St. Louis, MO; lot 36°F-58851) was added and the samples were incubated for 1 h at room temperature. The resulting chromophore was measured by spectrophotometer at 414 nm.

### Elastase Functional Assay

The ability of macrophage supernatants to inhibit NE was determined by elastase functional assay. Aliquots of 100  $\mu\text{l}$  of serial dilutions of macrophage supernatant were incubated with 100  $\mu\text{l}$  of HNE (1  $\mu\text{g/ml}$ ) for 1 h. After incubation, elastase activity was measured by adding 100  $\mu\text{l}$  of test solution to 200  $\mu\text{l}$  of 0.2 mM of the methoxysuccinyl-L-alanyl-L-alanyl-L-prolyl-L-valine *p*-nitroanilide substrate in 0.1 M HEPES buffer at pH 7.5 containing 0.5 M NaCl and 10% dimethylsulfoxide. Absorbance at 414 nm before and 1 h after adding the samples was determined and change of absorbance at 414 nm was used as a measure of elastase activity.

### Partial Characterization of Macrophage Factor

To partially characterize the macrophage-derived factor, macrophage supernatants were treated in several ways: Macrophage supernatant (undiluted) was boiled at  $100^\circ\text{C}$  for 15 min. Lipid extraction was performed by adding ethylacetate to macrophage supernatant and mixing. Aqueous and lipid fractions were separated and both were assayed for elastase binding capacity. Macrophage supernatant was centrifuged at 20,000 *g* for 1 h. Macrophage supernatant was incubated with a 1:100 dilution of polyclonal goat anti-human  $\alpha_2$ -macroglobulin antibody for 3 h (M-5649, lot 18H4819; Sigma). Finally cycloheximide (25  $\mu\text{g/ml}$ ) was added to the macrophage cultures. Percent elastase binding inhibition was calculated as follows:

$$\frac{\text{Absorbance (RPMI)} - \text{Absorbance (Sample)}}{\text{Absorbance (RPMI)} - \text{Absorbance (Crude)}} \times 100 (\%).$$

### Sephadex G-150 Column Chromatography

A volume of 50 ml of AM culture supernatant was dialyzed against 5 L of distilled  $\text{H}_2\text{O}$  for 2 d, lyophilized, and resuspended in 2.5 ml of buffer. The concentrated macrophage supernatant was then filtered through a 0.45- $\mu\text{m}$  milipore filter (Gelman Science Inc., Ann Arbor, MI; lot 5915). Culture supernatants run in parallel were also incubated with HNE at a concentration of 15  $\mu\text{g/ml}$ . The samples were then loaded on a Sephadex G-150 column (1.5  $\times$  100 cm; Bio-Rad

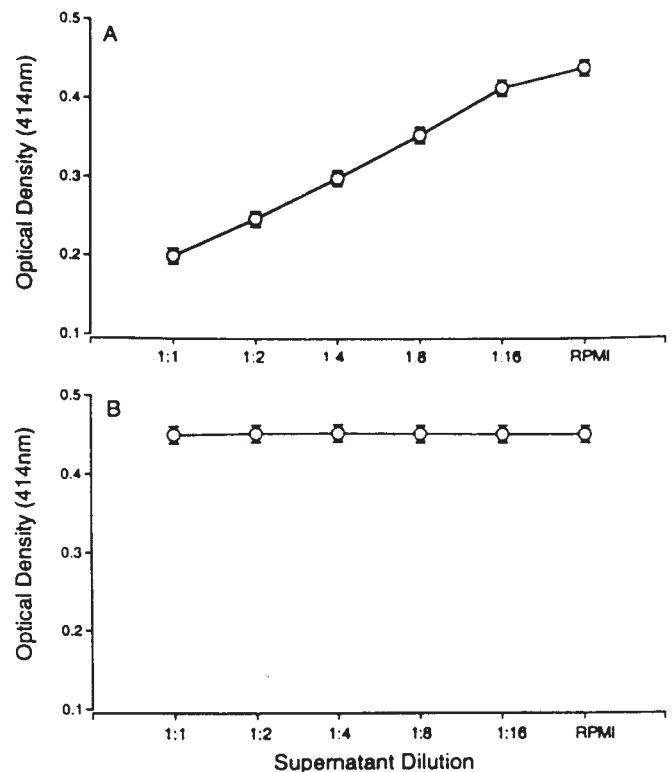
Laboratories, Richmond, CA) equilibrated with PBS (pH 7.4). The column was run at a flow rate of 25 ml/h, and 2-ml fractions were collected. In order to determine if the elastase activity synthesized *in vivo* could be separated into low- and high-molecular-weight fractions and if these fractions could be altered by various treatments, BALF, which had free elastase activity, were obtained from patients with pneumonia. The BALF was subjected to various treatments: It was sonicated for 30 s and then incubated with 100  $\mu\text{g/ml}$  deoxyribonuclease (DNase) for 1 h. Also, BALF was sonicated for 30 s, incubated with 100  $\mu\text{g/ml}$  (DNase) for 1 h and the lipid-inextractable fraction was recovered after mixing with ethylacetate. After these treatments, the solutions were filtered and loaded on a Sephadex G-150 column as previously described. Free elastase activity in G-150 column fractions was then measured as previously described.

### Quantification of Fibronectin and $\alpha_1$ -Protease Inhibitor ( $\alpha_1$ -PI)

Fibronectin and  $\alpha_1$ -PI in column fractions were measured by enzyme-linked immunosorbent assays (ELISA) as molecular weight standards (10).

### Statistical Analysis

Data are presented as mean values  $\pm$  SD. Student's *t* test was used to evaluate statistical difference. A *p* value of 0.05 or less was considered statistically significant.



**Figure 1.** (A) Inhibition of elastase binding to elastin by supernatants from cultured macrophages from normal smokers. *Vertical axis:* chromophore generated, representing active elastase bound to insoluble matrix. *Horizontal axis:* macrophage supernatant dilution. Each data point represents mean  $\pm$  SE of 10 independent experiments. (B) Supernatants from cultured macrophages do not alter NE functional activity. *Vertical axis:* chromophore generated, representing elastase activity. *Horizontal axis:* macrophage supernatant dilution. Each data point represents mean  $\pm$  SE of three independent experiments.

## RESULTS

### Effect of Macrophage Supernatant on Elastase Binding to Elastin

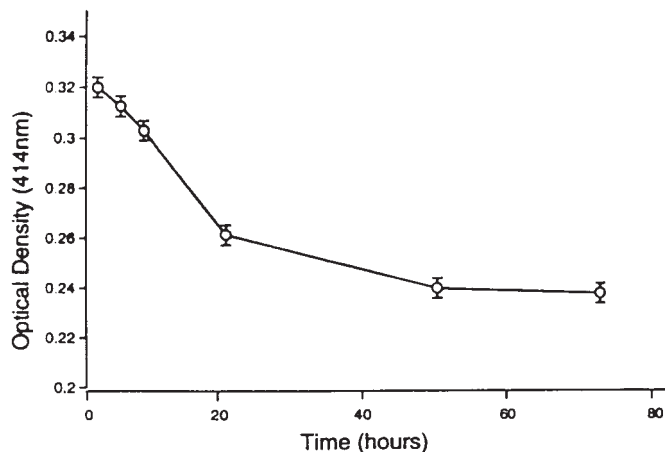
Elastase binding to elastin was inhibited by AM supernatant in a dose-dependent manner (Figure 1A). Binding to type IV collagen was similarly inhibited (data not shown). Importantly, elastase activity per se was not altered after incubation with macrophage supernatant (Figure 1B). This implies that lack of detectable bound elastase activity was caused by inhibition of binding rather than inhibition of elastase activity. Accumulation of the macrophage factor in supernatant media was linear in 24 h, and increased accumulation was observed in the macrophage supernatant for at least 72 h (Figure 2). Macrophage supernatants had neither detectable elastase activity nor NE activity inhibitory capacity under these conditions (data not shown).

### Measurement of $\alpha_1$ -PI and $\alpha_2$ -Macroglobulin in Macrophage Culture Supernatants

Because AM are capable of releasing  $\alpha_1$ -PI and  $\alpha_2$ -macroglobulin which both can inhibit elastase activity, the concentration of these protease inhibitors was assayed in macrophage culture supernatants. The concentration of  $\alpha_1$ -PI was  $112.8 \pm 33.6$  ng/ml (mean  $\pm$  SEM) and  $439.2 \pm 57.6$  ng/ml from macrophage culture supernatants obtained from nonsmokers and smokers, respectively. Correspondingly, the concentration of  $\alpha_2$ -macroglobulin was  $17.0 \pm 9.3$  and  $91.2 \pm 24.8$  ng/ml from nonsmokers and smokers, respectively. However, the concentration of these protease inhibitors required to inhibit elastase activity *in vitro* was considerably higher. Thus  $2 \mu\text{g/ml}$  of  $\alpha_1$ -PI and  $16 \mu\text{g/ml}$  of  $\alpha_2$ -macroglobulin were required for 50% inhibition of  $1 \mu\text{g/ml}$  of NE.

### Partial Characterization of Macrophage Factor

The macrophage factor was relatively heat-stable, but lost its activity after ethylacetate extraction (Figure 3). Inhibitory activity could be recovered in neither the aqueous nor the lipid fraction after extraction. In addition, after ultracentrifugation, inhibitory activity in the supernatant was significantly decreased (Figure 3). Most of the activity remained after incu-



**Figure 2.** Time course of macrophage supernatant capacity to inhibit elastase binding to elastin. Macrophages were cultured at  $1 \times 10^6$  cells/ml in RPMI medium. Supernatant media were harvested at different times and assayed for their capacity to inhibit neutrophil binding to elastin. Each data point represents mean  $\pm$  SE of three independent experiments.

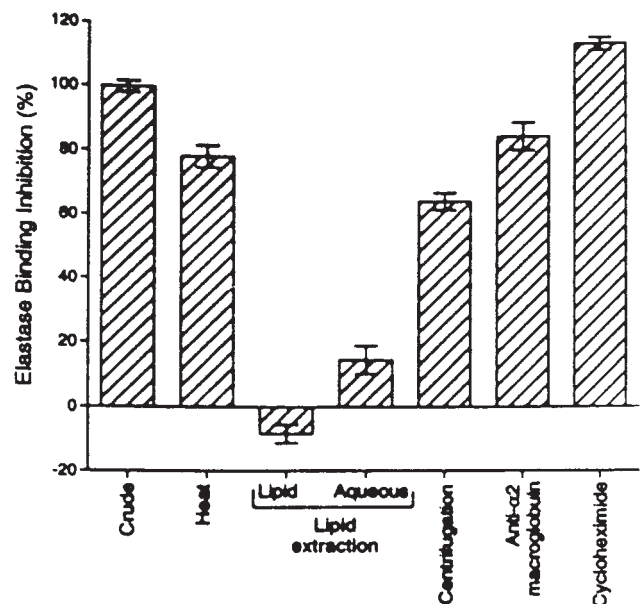
bation of the supernatant with anti- $\alpha_2$ -macroglobulin antibody. Cycloheximide did not decrease the inhibitory activity.

### Column Chromatography

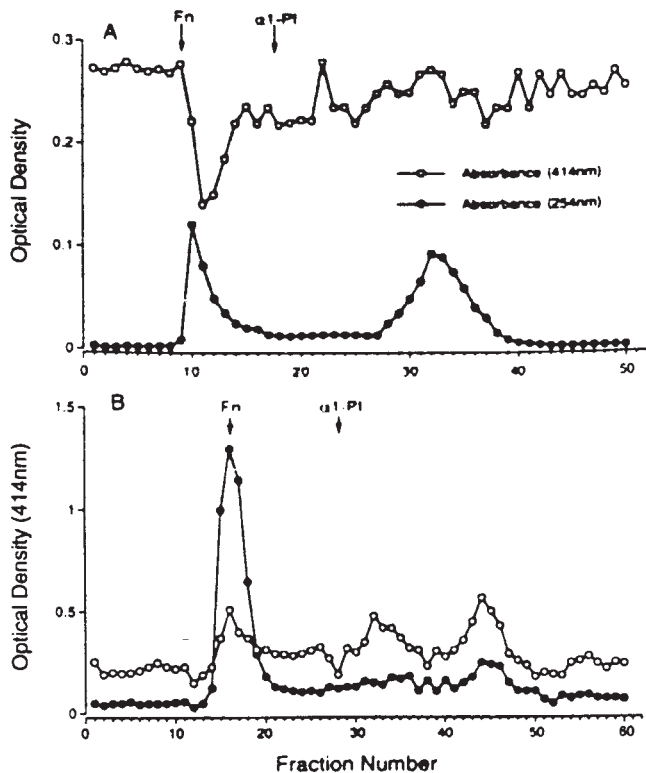
Sephadex G-150 column chromatography indicated that the inhibitor had a heterogenous high molecular weight: the peak of activity was near 440,000, but distributed to 54,000 (Figure 4A). When NE was added to macrophage supernatants, functional enzyme was recovered with a molecular weight distribution similar to that of the binding inhibitor (Figure 4B). This suggests a factor or factors that could bind to HNE and make a high-molecular-weight complex. Moreover, while the high-molecular-weight elastase retained functional activity, it did not bind elastin well. The ratio of elastin binding to functional elastase activity was significantly lower in the high-molecular-weight fractions 14, 15, and 16 (0.36, 0.43, and 0.37) than in the lower molecular weight fractions 44, 45, and 46 (2.7, 2.2, and 2.1,  $p < 0.05$ ).

### Ability of Smokers' and Nonsmokers' Macrophages to Release Inhibitory Factor *In Vitro*

The capacity of supernatants from cultured macrophages to inhibit elastase binding to elastin was assayed in AM supernatants obtained from nonsmokers and smokers. Macrophage supernatants from smokers had significantly ( $p < 0.001$ ) higher capacity to inhibit elastase binding to elastin than those from nonsmokers (Figure 5).



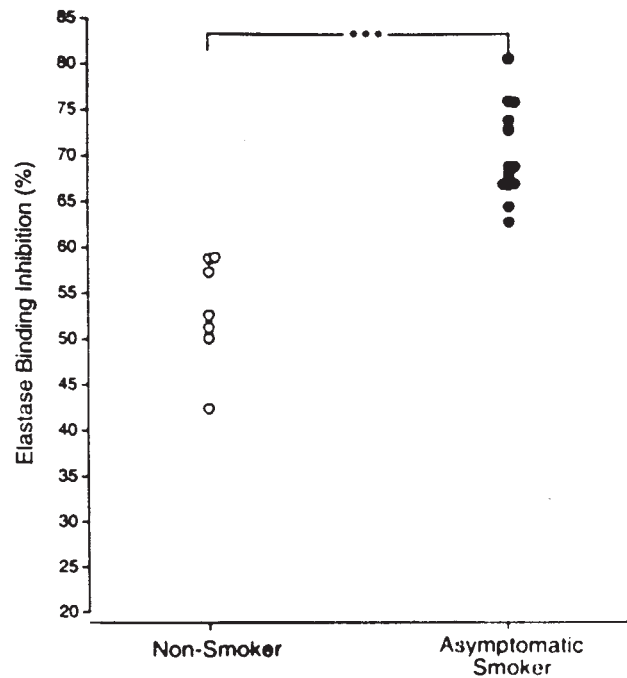
**Figure 3.** Partial characterization of the macrophage activity. Macrophage supernatants were treated in several ways: Macrophage supernatant was boiled at  $100^\circ\text{C}$  for 15 min. Lipid extraction was performed by adding ethylacetate to macrophage supernatant and mixed. Aqueous and lipid fractions were separated. Macrophage supernatant was ultracentrifuged at  $20,000\text{ g}$  for 1 h. Percent elastase binding inhibition was then calculated. \*In addition, the supernatants were incubated with antibodies against  $\alpha_2$ -macroglobulin as well as treated with cycloheximide. Each bar represents standard deviation of four independent experiments. \*\*There was a statistical significant difference ( $p < 0.001$ ) between all treatment groups and "crude."



**Figure 4.** (A) Sephadex G-150 molecular sieve column chromatography. To characterize macrophage factor further, 20 ml of macrophage supernatant obtained from a normal smoker was dialyzed, lyophilized, and resuspended in 1 ml of PBS. The concentrated supernatant was then filtered by 0.45- $\mu$ m milipore filter and loaded on Sephadex G-150 column. Elastase binding to elastin (*open circles*) was determined functionally and is expressed as absorbance at 414 nm. Absorbance at 254 nm as a measure of protein content was also determined for each fraction (*closed circles*). In addition, fibronectin (molecular weight: 440,000) and  $\alpha_1$ -PI (molecular weight: 54,000) were measured in each fraction (ELISA) and the peaks are shown as molecular weight standards. (B) Elastase activity and elastase binding to elastin in macrophage supernatant mixed with NE. To evaluate the mechanism of inhibition of elastase binding to elastin by AM factor, concentrated macrophage supernatant and HNE (final concentration 15  $\mu$ g/ml) were mixed and loaded on a Sephadex G-150 column. Both functional elastase activity (*closed circles*) and elastase binding to elastin (*open circles*) were evaluated in each fraction. Fibronectin and  $\alpha_1$ -PI in each fraction were measured by ELISA as internal molecular weight standards.

#### High-molecular-weight Elastase in BALF

BALF from patients with pneumonia who had measurable elastase activity were used. Both "high-molecular-weight" elastase and "low-molecular-weight" elastase were present in these fluids (Figure 6A). Free DNA is present in purulent samples, and because binding to DNA could result in elastase eluting together with high-molecular-weight samples, aliquots of BALF were treated with DNase. However, after DNase treatment most of the elastase in the high-molecular-weight column fractions still remained, suggesting that binding of elastase to DNA was not the cause of the size fractionation (Figure 6B). In contrast, most of the elastase was present in the low-molecular-weight fractions after ethylacetate extraction (Figure 6C).

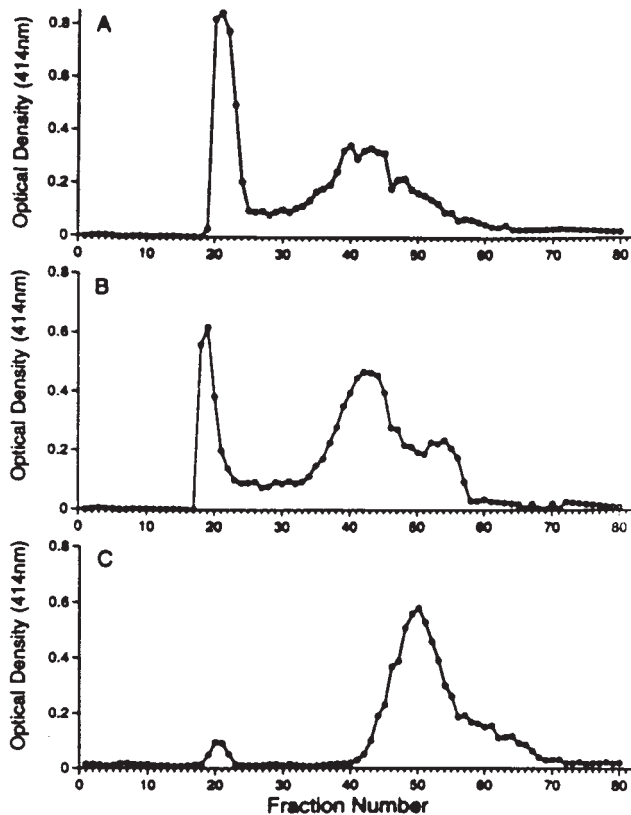


**Figure 5.** Quantification of binding inhibition in cultured macrophage supernatants from nonsmokers and smokers. Cells obtained by BAL from seven normal nonsmokers and 13 normal smokers were cultured for 24 h in RPMI without serum. Percent elastase binding inhibition in the culture supernatant was determined as described in METHODS. All values represent the mean of the three determinations. \*\*\* $p < 0.001$ .

## DISCUSSION

In the current study, we demonstrate that macrophage-derived factors can inhibit elastase binding to elastin. The factors (probably lipid aggregates) can bind to HNE and make a high-molecular-weight complex. This "high-molecular-weight" elastase binds to elastin significantly less than does native elastase, although it remains functional. Culture supernatants from AM from smokers contain more of this factor than do supernatants of macrophages from nonsmokers. Finally, we were also able to detect a "high-molecular-weight" elastase in BALF from patients with pneumonia in a form that was sensitive to extraction but not to DNase treatment.

Our results show that macrophage factors can interact with HNE and inhibit its binding to elastin without inhibiting its functional activity. This macrophage factor had a heterogeneous molecular weight and seemed to be, at least in part, a lipid. It was heat-stable, it was partially lost after ultracentrifugation, and it was not recovered after ethylacetate extraction in either the aqueous or the lipid fraction. The inhibitor was not a protein, because cycloheximide added to the macrophage cultures did not decrease the inhibitory activity. Macrophages can produce several factors that can interact with NE (11, 12). These factors include the protease inhibitors,  $\alpha_1$ -PI (13, 14), and  $\alpha_2$ -macroglobulin (15). Both of these factors can inhibit elastase binding to elastin as detected functionally in this study but the amounts required are large. The concentrations required to inhibit the concentration of elastase used in the present study (1  $\mu$ g/ml) are considerably higher than those produced by AM under the conditions used. Thus, it is unlikely that either of these inhibitors accounts for the decreased binding of elastase to elastin detected in the macrophage su-



**Figure 6.** High-molecular-weight-elastase in BALF. One ml of concentrated BALF which had free elastase activity obtained from a patient with pneumonia was treated in several ways (A: sonication only; B: sonication + DNase treatment; C: sonication + DNase treatment + ethylacetate extraction) and loaded on a Sephadex G-150 column. Functional elastase activity was evaluated in each fraction.

pernatants. In addition, most of the inhibitory activity remained when the macrophage supernatants were incubated with antibodies directed toward  $\alpha_2$ -macroglobulin.

Macrophages can bind and internalize NE by virtue of a specific membrane receptor for this and other neutrophil glycoprotein enzymes (16, 17). An alternative mechanism by which macrophages could take up elastase could be by the uptake of elastase-lipid complexes. In this regard, AM can phagocytose liposomes as well as release them (18). The macrophage-derived lipid described in the current study may serve as a sink to bind and trap elastase in the extracellular milieu so that it can subsequently be cleared by macrophages.

Macrophages accumulate in centrilobular zones of asymptomatic cigarette smokers prior to the onset of significant tissue destruction in emphysema (6). In the present study, human smokers' macrophages released significantly higher amounts of the inhibitor than nonsmokers'. Once bound to elastin, NE may not be subject to inhibition by  $\alpha_1$ -PI. Thus, binding of elastase to elastin may be a crucial first step in the development of emphysema. This suggests that macrophages may play a protective role by secreting this inhibitor of binding. The ability to augment the release of this inhibitor may be an important protective mechanism in some smokers.

High-molecular-weight elastase was also detected *in vivo*. Thus, BALF which had free elastase activity, obtained from patients with pneumonia, contained elastase in a high-molecular-weight complex. Although it has been reported that alveo-

lar fluid NE activity in the adult respiratory distress syndrome is complexed to  $\alpha_2$ -macroglobulin (19), this "high-molecular-weight" elastase had properties similar to the macrophage product: it was not present in the lipid-inextractable fraction after ethylacetate extraction and decreased its activity after ultracentrifugation, suggesting that the high-molecular-weight elastase has similar biochemical properties to the elastase-macrophage factor complex. It has been reported that DNA is capable of binding to HNE. Furthermore, DNA also has elastase inhibitory activity (20, 21). DNase treatment, however, did not disrupt the "high-molecular-weight" elastase, in contrast to lipid extraction. Taken together, the "high molecular elastase" seems to be complexed *in vivo* in the lower respiratory tract, which raises the possibility that these complexes may play a protective role.

While speculative, the ability of elastase to bind to lipid components in the lower respiratory tract has several possible functional implications. A high-molecular-weight elastase-lipid complex might remain in the surfactant layer within the alveolar or airway lumen and would not be able to penetrate into the interstitial tissue. The high-molecular-weight elastase-lipid complex, however, retains enzymatic activity. Thus, the formation of these complexes provides a mechanism to maintain a high concentration of elastase in the lipid phase of the lower respiratory tract lining fluid. Elastase in the lipid phase may be well positioned to kill bacteria in the airspace without being able to destroy the host interstitium. This partitioning of elastase may explain the long-standing observation that patients with acute bacterial pneumonia, even though free elastolytic activity can be detected within the airspace, do not usually suffer destruction of lung tissue and development of emphysema. Finally, partitioning of elastase into a lipid-bound phase may represent an efficient means by which elastase is cleared from the lower respiratory tract, either by clearance by the mucociliary elevator, or by phagocytosis.

In conclusion, macrophages release a factor or factors that can interact with NE and inhibit elastase binding to elastin. This macrophage-derived factor appears to be a lipid which may be important in protecting the lung from elastinolysis by NE.

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